50 times as great as when [^{14}C]acetate is the substrate.

4. The previously reported atypical labelling pattern in lycopene synthesized by intact tomatoes (Zabin, 1957) was probably the result of the use of an inadequate method of degradation of the labelled pigment.

We wish to thank Hoffmann-La-Roche and Co. (Dr O. Isler) for a generous gift of synthetic lycopene. One of us (G.D.B.) is grateful to the Medical Research Council for a scholarship.

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Studies in Carotenogenesis

26. THE INCORPORATION OF [^{14}C]ACETATE, [^{14}C]MEVALONATE AND $^{14}\text{CO}_2$ INTO β -CAROTENE BY THE FUNGUS *PHYCOMYCES BLAKESLEEANUS**

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(Received 12 November 1959)

When mevalonic acid ($\beta\beta$ -dihydroxy- β -methylvaleric acid) was found to be an extremely effective precursor of cholesterol in liver preparations (see Popják, 1958; Wolstenholme & O'Connor, 1959), it was important to ascertain whether it was also an effective carotenoid precursor. The major part of this paper is concerned with experiments which have been carried out on the incorporation of DL-[2- ^{14}C]mevalonate into β -carotene by the fungus *Phycomyces blakesleeanus*, and to compare it with [2- ^{14}C]acetate.

The incorporation of $^{14}\text{CO}_2$ into β -carotene by *P. blakesleeanus* in the presence and absence of leucine has also been investigated, in an attempt to explain the results of Mackinney and his colleagues on the incorporation of [^{14}C]leucine into β -carotene in the same organism (Wuhrmann, Yokoyama & Chichester, 1957; Yokoyama, Chichester, Nakayama, Lukton & Mackinney, 1957; Mackinney,

Chandler & Lukton, 1958; Chichester, Yokoyama, Nakayama, Lukton & Mackinney, 1959).

The results of part of this investigation have been briefly reported previously (Braithwaite & Goodwin, 1957a, b; Goodwin, 1959a, b).

EXPERIMENTAL

Cultural conditions. The strain of *P. blakesleeanus* used in this investigation was that originally used by Garton, Goodwin & Lijinsky (1951). It was maintained on slopes of Sabouraud agar (Oxoid). The methods used for sterilizing and inoculating liquid media have been described in detail by Garton *et al.* (1951). Experimental cultures were generally carried out in 250 ml. Erlenmeyer flasks containing 30 ml. of the standard medium of Garton *et al.* (1951). The flasks were continuously shaken in a rotary shaker and incubated at 28° in an illuminated cabinet (Garton *et al.* 1951). For the experiments with $^{14}\text{CO}_2$, mats of *P. blakesleeanus* were cultured in Petri dishes.

Extraction, purification and crystallization of β -carotene. The extraction and purification of β -carotene from the harvested mycelia have been described (Garton *et al.* 1951; Goodwin, 1952). Unlabelled synthetic β -carotene was

* Part 25: Braithwaite & Goodwin (1960).

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added to the chromatographically-pure labelled β -carotene and the mixture crystallized to constant specific activity (Goodwin, 1958a).

Determination of β -carotene. The amount of β -carotene present in various fractions was determined with a Unicam SP. 600 photoelectric spectrophotometer (Goodwin, 1955).

Extraction, purification and determination of partly saturated polyenes. In the presence of diphenylamine, which inhibits carotenogenesis, *P. blakesleeanus* accumulates a number of partly saturated polyenes, such as phytoene, phytofluene and ζ -carotene. These were purified chromatographically and assayed spectrophotometrically (Goodwin, 1952, 1955).

Degradation of β -carotene. β -Carotene was oxidized with alkaline permanganate by the technique of Karrer, Helfenstein, Wehrli & Wettstein (1930) adapted to small quantities (Braithwaite & Goodwin, 1960). The acetic acid formed from C-9, C-9', C-13, C-13' and the adjoining methyl groups of β -carotene (I) was degraded by the Schmidt

Table 1. *Incorporation of [2- 14 C]acetate into β -carotene by *Phycomyces blakesleeanus* in the presence and absence of DL-mevalonate and L-leucine*

[2- 14 C]Acetate (0.47 μ mole, 17.2×10^6 counts/min.) was added with or without concomitant addition of DL-mevalonate (12 μ moles) or L-leucine (12 μ moles) to 48 hr. cultures on basal medium; the cultures were shaken at 28° and the cells harvested 48 hr. later.

Addition	Specific activity of β -carotene* (counts/min./ μ mole)
[2- 14 C]Acetate alone	394 000
	383 000
[2- 14 C]Acetate + DL-mevalonate	241 000
	254 000
[2- 14 C]Acetate + L-leucine	231 000

* After three recrystallizations, specific activity referred to β -carotene originally present.

reaction, by a modification of the method of Schuerch & Huntress (1949) (Braithwaite & Goodwin, 1960).

Radioactive materials. [1- 14 C]Acetate, [2- 14 C]acetate and Ba 14 CO $_3$ were purchased from The Radiochemical Centre, Amersham, Bucks. DL-[2- 14 C]Mevalonate was kindly synthesized by Dr R. H. Cornforth.

Assay of 14 C. All measurements were made with a proportional gas-flow counter (Nuclear Measurements, Chicago; model PC-3); details have been described (Braithwaite & Goodwin, 1960).

RESULTS AND DISCUSSION

*Effect of mevalonate and leucine on the incorporation of [2- 14 C]acetate into β -carotene by *Phycomyces blakesleeanus**

Experiments were carried out to determine whether unlabelled mevalonate would decrease the incorporation of [2- 14 C]acetate into β -carotene by *P. blakesleeanus*. Similar experiments were carried out simultaneously with leucine, which is known to be carotenogenic (Goodwin & Lijinsky, 1952) by virtue of its ability to provide a key intermediate (Chichester *et al.* 1959). The mould was grown in shake culture (15 $\times$ 250 ml. Erlenmeyer flasks containing 30 ml. of medium) under our standard conditions, and as soon as β -carotene synthesis became apparent (usually after 48–60 hr.) the cells were bulked and divided into five groups with three flasks in each group; this reduced inter-flask variation to a minimum. [2- 14 C]Acetate was added to two groups, [2- 14 C]acetate and unlabelled DL-

mevalonate to two more, and [2- 14 C]acetate and L-leucine to the fifth. The results of one experiment in this series are recorded in Table 1; they show that, under the specified conditions, leucine and mevalonate decrease the incorporation of labelled acetate into β -carotene by about 40 %.

In isotope-dilution experiments with whole cells a negative result does not necessarily mean that the compound under examination is not a precursor, because permeability barriers may come into play. On the other hand, however, positive results, such as those obtained here, still do not prove that a compound is a precursor; it may be merely acting as a competitive inhibitor; indeed this has been demonstrated with β -hydroxy- β -methylglutaric acid and other possible steroid precursors in steroid synthesis (see Popják, 1958). As leucine, which is known to provide an active precursor of β -carotene, decreases the incorporation of [2- 14 C]-acetate to the same extent as mevalonate, the present results strongly suggest, but do not prove, that mevalonate is a precursor of β -carotene. Direct proof came from experiments with [2- 14 C]-mevalonate.

*Incorporation of D-[2- 14 C]mevalonate and [2- 14 C]acetate into β -carotene by *Phycomyces blakesleeanus**

Table 2 demonstrates that DL-[2- 14 C]mevalonate is incorporated into β -carotene by *P. blakesleeanus*. These experiments were first briefly reported by Braithwaite & Goodwin (1957b), and they have

Table 2. Incorporation of DL-[2- ^{14}C]mevalonate and [2- ^{14}C]acetate into β -carotene by *Phycomyces blakesleeanus*

The cultural conditions are as described in Table 1. Labelled substrates were added 48 hr. after inoculation and the cells harvested after a further 48 hr.; the specific activity of [2- ^{14}C]acetate was 5.0×10^{12} counts/min./mole, and that of DL-[2- ^{14}C]mevalonate was 2.2×10^{10} counts/min./mole.

	[2- ^{14}C]Acetate	[2- ^{14}C]Mevalonate
Total radioactivity (counts/min.)	16.1×10^6	2.01×10^{10} *
Counts utilized	16×10^6	0.63×10^6
Percentage utilization	100	31
Specific activity of β -carotene originally present (counts/min./ μ mole)	115 000	22 500
Total counts in β -carotene	10 600	2 200
% Incorporation into carotene of counts utilized	0.065	0.34

* Twice this number of counts was actually added (as DL-mevalonate); Wolf *et al.* (1957) have shown that DL-mevalonate has only one-half the biological activity of the natural compound obtained from distiller's dried solubles.

since been confirmed in *P. blakesleeanus* by Mackinney *et al.* (1958) and in *Mucor hiemalis* by Grob (1957). Table 2 also indicates that in this experiment not all the added mevalonate was utilized by *P. blakesleeanus* but that added acetate was completely utilized. In later experiments greater utilization of mevalonate was obtained if it was added (a) at the time of inoculation, or (b) not

method of identifying the carbon atoms of the isoprenoid repeating unit is

The two possibilities are indicated formally in (II) and (III) (C-2 is starred).

as the lactone but as the salt, dissolved in 0.1N-NaOH. However, in both cases the percentage of mevalonate utilized which appeared in the β -carotene was essentially the same as in the experiment recorded in Table 2. When the differential utilization of mevalonate and acetate is taken into account, then, based on total counts incorporated, mevalonate is five times as effective as acetate as a β -carotene precursor. However, as three acetate-methyl groups are incorporated into each C_6 unit for every one C-2 of mevalonate utilized, the true relative efficiency is three times this figure. Thus under our conditions mevalonate is fifteen times more effectively incorporated into β -carotene than is acetate.

The next point to be investigated was whether C-2 of mevalonate maintained its individuality during condensation to form β -carotene, and, if so, whether it appeared in C- w' or C- w . The accepted

If randomization of C-2 did occur then both C- w' and C- w would be labelled. This problem was settled by partial degradation of the β -carotene produced in the presence of DL-[2- ^{14}C]mevalonate. The acetic acid [from C-9, C-9', C-13, C-13' and the attached methyl groups; see (I)] which was liberated by alkaline permanganate oxidation, contained C- w' and C- x of the isoprenoid units and was collected and examined. Since no significant labelling was found, it appears that the pathway leading from mevalonate to β -carotene proceeds formally according to scheme (II). Further, this mechanism requires that the labelling observed in the CO_2 liberated from the side chain of β -carotene during oxidation with alkaline permanganate is approximately equal to that in the dimethyl acid fraction (dimethylglutaric acid and dimethylsuccinic acid), arising from the ring if the loss of a little C-4 in producing dimethylsuccinic acid is

ignored. Thus the CO_2 and the dimethyl acids should each contain four carbon atoms arising from C-2 of mevalonate. Such equality of labelling was found in one experiment where the total activity in the dimethyl acids was 930 counts/min. and, in the liberated CO_2 , 1160 counts/min.

These results show that the incorporation of DL-[2- ^{14}C]mevalonate into β -carotene follows the same pattern as that found in sterols (Popják, 1958) and other terpenoids (Wolstenholme & O'Connor, 1959) and it seems extremely likely that β -carotene and sterol biosynthesis follow a common pathway in the early stages. The elucidation of the mechanism by which a common intermediate is channelled into one or the other pathway is a key problem in this field.

*Incorporation of $^{14}\text{CO}_2$ into β -carotene by
Phycomyces blakesleeanus*

The discovery that leucine stimulated β -carotene synthesis in *P. blakesleeanus* (Goodwin & Lijinsky, 1952) suggested that the incorporation of $^{14}\text{CO}_2$ into β -carotene by this organism should be greater in the presence of this amino acid, because the conversion of leucine into β -hydroxy- β -methylglutaric acid and thus into mevalonate in animal tissues involves the fixation of CO_2 into β -methylcrotonyl-S·CoA (CoA·SH = coenzyme A) (Coon, Kupiecki, Dekker, Schlesinger & del Campillo, 1959), thus:

The results given in Table 3 demonstrate that no fixation of $^{14}\text{CO}_2$ into β -carotene occurs in *P. blakesleeanus* cultured on our basal medium; if leucine is added to the medium, however, incorporation of the label into β -carotene takes place. This indicates that the pathway of leucine metabolism in *P. blakesleeanus* is probably the same as that just indicated for animal tissues. However, Durr, Rudney & Ferguson (1959) have shown that, in the conversion of β -hydroxy- β -methylglutaryl-S·CoA into mevalonate, it is the carbon atom carrying the CoA residue which is reduced to the alcohol group of mevalonate. This means that the

Table 3. *Incorporation of $^{14}\text{CO}_2$ into β -carotene by Phycomyces blakesleeanus*

48 hr. cultures, on basal medium in Petri dishes, were transferred for 48 hr. to an atmosphere (vol. 5 l.) containing 0.3% of $^{14}\text{CO}_2$. Total activity 66×10^5 counts/min., at 28° .

Nitrogen source in medium	Specific activity of β -carotene (counts/min./ μmole)*
0.2% L-Asparagine	0
0.1% L-Asparagine + 0.1% L-leucine†	11 000

* Twice recrystallized; specific activity referred to β -carotene originally present.

† In order to maintain normal growth rate, the nitrogen source was not completely replaced by leucine.

C atom arising from CO_2 becomes C-1 of mevalonate which is lost on polymerization (Wolstenholme & O'Connor, 1959). The appearance of the label from $^{14}\text{CO}_2$ in β -carotene in the presence of leucine can be explained in two ways: (a) (Goodwin, 1959a) and (b) (Lynen, 1959) (the C atom arising from CO_2 is starred). The objection to the first proposal is that a β -hydroxy- β -methylglutarate-activating enzyme has not yet been unequivocally detected in any tissue. Further, the second proposal can account rather more readily for the observations of Mackinney and Chichester and their colleagues on the incorporation of leucine labelled with ^{14}C in

different positions into β -carotene by *P. blakesleeanus*. As would be expected, C-1 of leucine is not incorporated (Chichester, Nakayama, Mackinney & Goodwin, 1955) and C-2 and C-3 are only weakly incorporated (Chichester *et al.* 1955; Yokoyama *et al.* 1957); according to scheme (b) these two carbon atoms give rise to acetyl-S·CoA, and it is a reasonable assumption that acetyl-S·CoA is in equilibrium with a larger endogenous pool than is acetoacetate or acetoacetyl-S·CoA. C-4 and C-5 of leucine, on the other hand, are strongly incorporated (Yokoyama *et al.* 1957; Wuhrmann *et al.* 1957; Chichester *et al.* 1959); this is because

SUMMARY

1. The incorporation of [2-¹⁴C]acetate into β -carotene by *P. blakesleeanus* is decreased by DL-mevalonate and by L-leucine.

2. DL-[2-¹⁴C]Mevalonate is incorporated into β -carotene by *P. blakesleeanus* some fifteen times more efficiently than [2-¹⁴C]acetate.

3. The C-2 of mevalonate retains its individuality when incorporated into β -carotene and occurs only in C-*w* of the isoprenoid residue.

4. *P. blakesleeanus* cultured on our basal medium, with asparagine as the nitrogen source, does not fix CO₂ into β -carotene; however, when leucine is present in the medium ¹⁴CO₂ appears in the β -carotene. An explanation of this observation is offered.

5. Labelled β -carotene is synthesized by washed suspensions of *P. blakesleeanus* grown in the presence of diphenylamine and DL-[2-¹⁴C]mevalonate.

We thank Hoffman-La-Roche and Co. (Switzerland) for a gift of synthetic β -carotene (Dr O. Isler) and Roche Products Ltd. (England) for the gift of a shaking machine (Dr A. L. Morrison). Dr Rita Cornforth kindly synthesized the DL-[2-¹⁴C]mevalonate used in the investigation. One of us (G.D.B.) participated in this work as the holder of a Medical Research Scholarship for training in Research Methods.

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Biochem. J. (1960) **76**, 10

Inhibition of Amylase Activity of Human Body Fluids by Citrate

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(Received 6 August 1959)

McGeachin, Dougherty, Hargan & Potter (1957) used the method of Van Loon, Likins & Seger (1952) to compare the amylase activities of plasma and serum

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prepared from the same blood sample. With human blood they found that, when oxalate or citrate was used as anticoagulant, the amylase activity of plasma was slightly lower than that of serum. With rat and dog blood they found no such difference.